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(54) Title: VACCINE AGAINST PATHOGENS OF MUCOSAE USING LIPOSOMES

(57) Abstract

Lipopolysaccharide (LPS) and outer-membrane protein (OMP) preparations of Bordetella pertussis were incorporated into multilamellar liposomes composed of soya bean derived phospholipids. After oral or intranasal vaccination of mice with the coated liposomes, specific antibody responses were detected in lung washes. However, a specific IgA response to LPS could be detected after immunisation with only the OMP-coated liposomes and not with the LPS-coated liposomes suggesting adjuvant activity bestowed by the proteins. The OMP-coated liposomes were significantly more effective in inducing an immune response than the OMP preparation alone. Responses were highest when mice were given a booster 30 days after primary immunisation. The maximum response occurred 20 days after the booster but specific antibody could still be detected 75 days after secondary immunisation. These results suggest that this liposome antigen delivery system has potential in stimulating secretory antibody responses which may be necessary to effectively protect against infection from B. pertussis.

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VACCINE AGAINST PATHOGENS OF MUCOSAE USING LIPOSOMES

PROBLEM

Pertussis is a severe respiratory disease which most commonly affects young infants and prior to the 1930s was a major cause of child morbidity and mortality. Since then, large scale parenteral vaccination with heat-killed whole-cell preparations of Bordetella pertussis (the causative bacterium) and improved socio-economic conditions have greatly reduced the incidence of pertussis in the developed countries However, in the past two decades there has been an upsurgence in the incidence of the disease following a reduced acceptance rate of the vaccine by parents mainly due to possible side effects which may be associated with pertussis vaccination. These range in severity from mild local reactions through persistent screaming to permanent brain damage. In some countries, doctors are reluctant to advise vaccination because of legal liability. In addition, vaccines can vary enormously between manufacturers and not all vaccine preparations are protective.

In recent years, the major thrust of pertussis research has been to develop defined acellular vaccines with reduced reactogenicity and greater immunogenicity. However, this is hampered by lack of knowledge of the pathogenesis of pertussis, which is exceptionally complex, and the immunologically protective mechanisms which follow infection. Bordetella pertussis possesses several factors which have been implicated in pathogenesis but it is still controversial which of these are the protective antigens in humans and which factors are associated with the reactogenicity of the vaccine. Vaccines based on purified antigens, namely detoxified pertussis toxin and filamentous haemagglutinin, gave some protection in Swedish field trials but it is clear that these vaccines were contaminated with other antigens which may also play a role in protection. Thus a non-reactogenic highly efficacious vaccine is still not available. Alternative strategies thus need to be explored. We have examined the possibility of using liposomes containing Bordetella pertussis antigens to stimulate airway immune responses after oral or nasal administration.

INTRODUCTION

The incidence of pertussis in the developed countries has been largely reduced through large scale parenteral vaccination with heat-killed whole-cell preparations of B. pertussis. However, pertussis is still a world-wide problem due to a reduced acceptance rate of the present vaccine following heavy publicity of the possible side effects associated with vaccination [48], variation in efficacy associated with the manufacturing of vaccines [19], and the lack of vaccination programs in the underdeveloped countries where 95 % of the incidence of pertussis occurs [34].

One possible reason for the lack of reproducible efficiency of vaccines is that parenteral administration of B. pertussis antigens may be insufficient to stimulate the necessary mucosal immune system required

as first line defense against infection. Indeed, some epidemiological evidence suggests that current vaccines protect more against the disease than against infection [11]. Oral or intranasal administration of antigens may not only give better protection but may also circumvent many of the side reactions associated with parenteral vaccination. However, antigens administered orally often induce a poor short-time immune response and require adjuvant to boost these responses.

Phospholipid bilayered vesicles (liposomes) have been used as delivery systems for a wide variety of biologically active substances to specific tissues and more recently have been used as immunological adjuvants to enhance the immune response to several bacterial and viral antigens [17]. Here we report the potential of liposomes as a delivery system to enhance the immunological response to B. pertussis surface antigens in the lungs.

In other words, Bordetella pertussis is the etiological agent of whooping cough, an infection of the human respiratory tract. The disease is particularly severe in young children and may lead to neurological disorders and death [62]. The impact of pertussis morbidity and mortality in health is usually underestimated due to underreporting; however 60 million cases and more than 500,000 deaths per year are estimated to have a pertussis aetiology or to be pertussis related [34]. The prevalence of pertussis in countries where vaccination is not mandatory is very high with 95 % of sera among 17 to 19 year old individuals containing antibodies against pertussis [15].

In the Developed Countries the incidence of whooping cough has been reduced largely by mass immunization with a heat-killed whole-cell vaccine [12]. The benefits associated with this vaccine clearly outweigh the risks of rare but severe adverse effects, but nevertheless public concern about safety and immunogenicity has resulted in decreased vaccination rates [22, 33]. Moreover, the efficacy of the whole cell vaccine has been questioned in a few countries such as the United States because although immunization rates are high, between 30,000-125,000 cases of pertussis have occurred annually [55]. Therefore, a new generation of non-toxic and highly immunogenic vaccines, composed of well-defined components are urgently needed. Several virulence factors of B. pertussis have been considered for inclusion in defined acellular vaccines and detoxified pertussis toxin (PT) and filamentous hemagglutinin (FHA) are prime candidates [29, 40]. Conventional subunit vaccines administered by parenteral route seem primarily elicit humoral immunity; mucosal antibody responses are poor. B. pertussis bacteria, however, infect through the respiratory tract mucosal membrane and specific immunoglobulin (Ig) A is elicited after natural infection [57]. Such antibodies may well protect the host from both colonization and disease. It would thus seem worthwhile to explore means of stimulating airways mucosal immunity and to evaluate its utility in protecting from infection and disease.

Liposomes have been successfully used as delivery systems for drugs, antigens, hormones, and genetic material [17]. Promising results have also been obtained following immunization with antigens entrapped in liposomes; the oral [18, 43, 45, 61] and parenteral [4, 8, 10, 24, 25, 46, 49, 52] routes have been used for antigens of parasites [4, 24, 25, 45, 49], virus [10, 38] and bacteria [8, 18, 43, 46, 52, 61]. Their potential as adjuvants have been demonstrated in several studies where the use of liposome-entrapped antigens resulted in protective immunity [4, 8, 18, 24, 25, 45, 49, 52], or at least cell-mediated [8] and humoral responses [10, 46]. The adjuvanticity of liposomes seems to depend on several factors including vesicle size and structure, lipid constitution, surface charge, antigen localization, the animal species immunized, route of immunization, and the distribution and number of lamellae. The association of orally administered antigens with liposomes enhance their absorption, targeting them to processing cells, and favors their presentation to T cells and uptake into regional lymphnodes. this process improves the induction of humoral, secretory and cell mediated immune responses [1, 2, 17, 58].

In the present report we describe the ability of liposome encapsidated FHA and PT to induce specific systemic or secretory immune responses following oral immunization of mice.

According to one embodiment, the invention concerns a vaccine against pathogens of mucosae where the vaccine consists of a liposome delivery system or contains said delivery system as reactive component where the delivery system consists of lipid vesicles or contains same characterized in that the lipid vesicles contain at least one antigen of the pathogens of mucosae incorporated into their outer membrane.

The vaccine according to the invention can be characterized in that the pathogen of mucosae is a strain of a species of Bordetella, especially B, pertussis, shigella or streptococcus.

Further, the vaccine according to the invention can be characterized in that the antigen is a preparation of the outer membrane protein (OMP) of Bordetella pertussis.

Further, the vaccine according to the invention can be characterized in that the antigen is a lipopolysaccharide (LPS) of Bordetella pertussis.

Further, the vaccine according to the invention can be characterized in that the antigen is filamentous haemaglutinin and/or pertussis-toxin of Bordetella pertussis.

Further, the vaccine according to the invention can be characterized in that both a preparation of the outer membrane protein (OMP) and a lipopolysaccharide (LPS) as well of Bordetella pertussis are contained as antigen.

Further, the vaccine according to the invention can be characterized in that the vaccine according to lipid-vesicles are small multilamelar phospholipid-vesicles based on soybean.

According to another embodiement, the invention concerns a process for the preparation of a vaccine according to the invention, characterized in that the antigen or the antigens are incorporated by means of the solvent dilution microcarrier technique into the lipid-vesicles.

Lipopolysaccharide (LPS) and outer-membrane protein (OMP) preparations of Bordetella pertussis were incorporated into multilamellar liposomes composed of soya bean derived phospholipids (Lyphasome^{TM}, Fountain Pharmaceuticals, U.S.A.). After oral or intra-nasal vaccination of mice with the coated liposomes, antigen-specific antibody responses were detected in lung washes. The OMP-coated liposomes were significantly more effective in inducing an immune response than the OMP preparation alone. Responses were highest when mice were given a booster 30 days after primary immunisation. The maximum response occurred 20 days after the booster but specific antibody could still be detected 75 days after secondary immunisation.

These results suggest that this liposome antigen delivery system has potential in stimulating secretory antibody responses which may be necessary to effectively protect against infection from B. pertussis and that it should also be applicable to the delivery of a variety of candidate protein and/or LPS antigens from B. pertussis.

Although liposomes have been used previously to elicit in-vivo antibody responses there are no reports of intra-nasal immunization or lung antibody responses following oral administration. This is of some importance since all present day whopping cough vaccines are administered parenterally and it is possible that this is insufficient to stimulate the mucosal immune system required as first line defense against colonization and subsequent infection. Indeed, some epidemiological evidence suggests that current vaccines protect more against the disease than against infection. Oral or intra-nasal administration of antigens may not only give better protection but may also circumvent the side reactions associated with parenteral vaccination such as the endotoxin shock due to small quantities of free LPS contaminating preparations of protein antigens. Our results show that liposome incorporated LPS does not result in endotoxin shock presumably by preventing insertion of lipidA portion of LPS into host cell membranes.

Such a delivery system may also be applicable to vaccinate against other bacterial pathogens that gain entry into the animal host via the mucosal route i.e. primarily the lungs or the intestinal tract.

Mice were orally vaccinated with liposome-entrapped filamentous hemagglutinin (FHA) and detoxified pertussis toxin (PT) of Bordetella pertussis. FHA- and PT-specific immunoglobulin (Ig) G was detected in serum, and both IgG and IgA in lung washes following the immunization. Antibody titres obtained in mice immunized with encapsidated antigen were significantly higher than those in mice immunized with unencapsidated FHA and PT, which demonstrated the adjuvanticity of the liposome carrier. The results indicate the potential usefulness of this approach to elicit immune responses against FHA and PT (and perhaps other pertussis antigens) in humans and its possible utility in large scale vaccination to protect both against B. pertussis infection and disease.

A detailed description of the invention based on figures and experimental parts A and B follows.

FIGURES

Figure 1.
SDS-PAGE of vesicles. Samples were analysed with an 11 % separating gel. 1, OMP-coated vesicles; 2, OMP preparation of B. pertussis; 3, whole cells of B. pertussis; 4, molecular weight markers.

Figure 2.
Electron microscopic examination of vesicles. Uncoated vesicles (A) and OMP-coated vesicles (C) were negatively stained with 4 % uranyl acetate; arrows indicate the multilamellar layers. B (uncoated vesicles) and D (OMP-coated vesicles) show vesicles after unidirectional metal-shadowing. OMP-coated vesicles were incubated with anti-OMP antibodies followed by Protein A-gold complexes (E, unstained vesicles; F, after metal shadowing); the gold particles (indicated by arrows) indicate the OMP material located at the outer surface of the OMP vesicles. In control experiments, OMP-coated vesicles were incubated with preimmune immunoglobulin followed by protein A-gold complexes (G) or with the protein A-gold complexes alone (H); no labeling of the OMP-coated vesicles was detectable. Bars represent 0.2 m.

Figure 3.
Antibody titres (anti-OMP) in lung washes of mice after intranasal vaccination with OMP-coated vesicles and unincorporated OMP preparation. Bars indicate standard deviation.

Figure 4.
The effect of dose of vaccine on antibody titres (anti-OMP) in lung washes of mice after intranasal vaccination with OMP-coated vesicles. Bars indicate standard deviation.

Figure 5.
Duration of antibody response after intranasal immunisation of mice with OMP-coated vesicles. Mice were vaccinated and killed at the days indicated and the anti-OMP titres from lung washes were determined. Bars indicate standard deviation.

Figure 6.
Western blot analysis of lung washes and serum. OMPs of B. pertussis were separated by SDS-PAGE with an 11 % separating gel and blotted. Strips 1 and 2 were incubated with pooled lung washes from mice immunised with OMP-coated vesicles, strips 3 and 4 with pooled lung washes from mice immunised with uncoated vesicles, strip 5 with pooled serum from mice immunised with OMP-coated vesicles and strip 6 with pooled serum from mice immunised with uncoated vesicles. Strips 1 and 3 were incubated with peroxidase conjugated anti-mouse IgA, as second antibody, and strips 2,4,5 and 6 with

anti-mouse IgG. The arrows point to the major 32 kd protein.

Figure 7.

Silver staining of LPS-coated vesicles. 1, LPS extracted from B. pertussis; 2, uncoated vesicles; 3, LPS-coated vesicles.

Figure 8.

Incorporation of FHA and PT into liposomes. Lane 1, molecular mass standards; lane 2, unencapsidated liposomes; lane 3, free FHA; lane 4, liposome-encapsidated FHA; lane 5, free PT; lane 6, liposome-encapsidated PT. FHA and PT subunits are indicated by arrows, as are the molecular masses of the standards in kilodaltons.

Figure 9.

Electron microscopic examination of liposomes. Uncoated liposomes negatively stained (A). BHA-coated liposomes negatively stained (B); after unidirectional metal-shadow (C); incubated with polyclonal anti-FHA antibodies and protein A-gold complexes without (D) and with (E) metal-shadow; incubated with preimmune serum and protein A-gold complexes (F). G: gold-particle; bars represent 0.2 um.

Figure 10.

Electron microscopic examination of PT-coated liposomes. PT-coated liposomes negatively stained with 4 % uranyl acetate, pH 4.5 (A); incubated with polyclonal anti-PT antibodies and protein A-gold complexes, metal-shadowed (B); control experiment, incubated with preimmune serum followed by protein A-gold complexes and metal-shadowed (C); post-embedding labeling, incubated with polyclonal anti-PT antibodies and protein A-gold complexes (D). G: gold-particle; bars represent 0.1 um.

Figure 11.

Levels of anti-FHA specific antibodies in sera (A) and lung washes (B) after oral immunization of mice with free and liposome-coated (L.C.) FHA. Standard deviations are indicated by vertical lines.

Figure 12.

Levels of anti-PT specific antibodies in sera (A) and lung washes (B) after oral immunization of mice with free and liposome-coated (L.C.) PT. Standard deviations are indicated by vertical lines.

EXPERIMENTAL PART A. PREPARATION OF VACCINES AND THEIR COMPONENTS

MATERIALS AND METHODS

Strains and cultivation

B. pertussis D300421 (serotype 1.2.3) [3] was grown on Bordet Gengou base (Difco) with 1 % (v/v) and 15 % (v/v) defibrinated horse blood. For large scale production, B. pertussis was grown in Modified Hornibrook medium [66].

Preparation of lipopolysaccharide

Two litres of 48 h B. pertussis culture were harvested by centrifugation and washed once with phosphate-buffered saline (PBS; NaCl 8 g/l, KCl 0.2 g/l, KH_2PO_4 0.2 g/l, $Na_2HPO_4.2H_2O$ 2.9 g/l, pH 7.4). The cells were resuspended to 75 ml with distilled water and incubated at 65 °C for 30 min. This was then mixed with an equal volume of 90 % (w/v) phenol and incubated at 65 °C for 45 min with constant mixing. The suspension was allowed to cool on ice and centrifuged at 10,000 x g for 10 min. The top aqueous layer was removed and centrifugated at 10,000 x g for 10 min to remove debris. The lipopolysaccharide (LPS) was precipitated by the addition of two volumes of ice cold acetone and incubation overnight. The precipitate was collected by centrifugation at 10,000 x g for 10 min at 4 °C and washed with 70 % acetone. The dried pellet was resuspended in 20 ml distilled water and ultracentrifugated at 100,000 x g for 2 h. Pellets were washed twice more with distilled water and then lyophilized.

Preparation of outer-membrane proteins

Two litres of 48 h B. pertussis culture were harvested by centrifugation and cells were washed once in PBS. Cells were resuspended in 50 ml PBS and heat-killed (56 °C for 30 min). Phenyl-methane-sulfonylfluoride (PMFS) was added to a final concentration of 0.1 mM and the cells were disrupted by sonication (ten one minute pulses on ice allowing one minute to cool inbetween pulses). Unbroken cells were removed by centrifugation at 5,000 x g for 10 min and envelopes were collected by centrifugation at 100,000 x g for 1 h. The envelopes were then resuspended in 20 ml 2 % triton X-100, 7.5 mM $MgCl_2$, 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) pH 7.4, allowed to stand for 1 h on ice, centrifuged at 100,000 x g for 1 h and washed once in the same buffer. The OMP preparation was then washed twice with distilled water. Protein concentrations were determined using a modification of the Lowry method [31].

Preparation of vesicles

Lyphazome™, a proprietary form of small multilamelar phospholipid vesicles, were prepared containing B. pertussis LPS and OMP preparations using the Solvent Dilution Microcarrier technique developed by

Fountain Pharmaceuticals Inc., [53] using purified soya bean phosphatides supplied by American Lecithin Company, New York. The composition of the purified lipid mixture was phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid and neutral lipids in an approximate ratio of 8:1:0.7:0.3.

Immunisation of mice

Four to five week old female Balb/c mice were immunised in groups of five. Mice immunised intranasally were anaesthetized by ether and 50 ul of vaccine dilution, in PBS, was deposited on the external nares and allowed to be enhaled. For mice immunised orally, vaccine was diluted appropriately in PBS and an equal volume of 3 % sodium bicarbonate in PBS (pH 8.0) was added just prior to immunisation to neutralise gastric acidity. Mice, deprived of water for 6 - 8 h, were gently fed with 50 ul. Unless otherwise stated, mice were immunised on days 1 and 4 and given a booster on day 30. One dose consisted of 4 ulg protein for OMP-coated vesicles, and 15 ug dry weight for LPS-coated vesicles and uncoated vesicles. Mice were killed ten days after booster, unless otherwise stated, by cervical dislocation and bled from the brachial artery. Lung washes were obtained by cannulating the trachea with a syringe and filling and emptying the lungs four times with 0.7 ml ice cold PBS with 0.1 mM PMFS. From each mouse about 0.5 ml was recovered, centrifugated at 4 °C at 10,000 x g for 10 min to remove debris and stored at - 20 °C.

Antiserum to OMP

OMP preparation was emmulsified at a ratio of 1:1 with Freunds incomplete adjuvant in a final volume of 1 ml and a three month Chinchilla rabbit was injected subcutaneously and intramuscularly on day 1 (200 ug), day 30 (100 ug) and day 40 (100 ug). The rabbit was killed on day 50 and immunoglobulin was purified from the obtained serum by protein A-sepharose CL4B chromatography.

SDS-PAGE and Western blot analysis

Proteins and LPS were electrophoretically separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [30]. Proteins were stained by commassie blue and LPS by silver staining [59]. Western blotting was carried out as previously described [5]. Briefly proteins were transferred to nitrocellulose using a semi-dry transfer cell with 25 mM

Tris, 192 mM glycine, 20 % methanol pH 8.3 as transfer buffer and 5 % bovine serum albumin in PBS as a blocking agent. Blocked membranes were incubated for 1 h at 37 °C with either lung washes, diluted 1 in 4 in PBS, or serum sample diluted 1 in 10 in PBS. After washing three times with PBS, the membranes were incubated with peroxidase conjugated goat anti-mouse IgA (Southern Biotechnology Associates Inc.) or anti-mouse IgG (Jackson Immunoresearch Laboratories) for 1 h at 37 °C. Membranes were washed three times in PBS and developed using 4-chloro-1-naphthol as a substrate.

ELISA

Microtitre plates (Nunc Maxisorp) were coated overnight at 4 °C with either OMP in O.1 M $NaHC0_3$ pH 9.6 (5 ug in 50 ul per well), or LPS in 50 mM Tris HCl pH 9.6, 20 mM $MgCl_2$ (I mg in 50 ul per well). The plates were washed and blocked with 10 % fetal calf serum (FCS) in PBS (IOO ul per well) for 1 h at 37 °C. After washing, plates were incubated with various dilutions of serum or lung washes in 10 % FCS in PBS (50 ul per well) for 1 h at 37 °C. Plates were washed and 50 ul (per well) of peroxidase conjugated goat anti-mouse IgA or anti IgG diluted 1 in 1000 in 10 % FCS in PBS was added. Plates were incubated as before and after washing plates were developed by addition of 50 ul (per well) of substrate (0.25 M citric acid, 0.25 M Na_2HPO_4, 0.0015 % H_2O_2, 0.3 mg ml^{-1} O phenyl-diamine dihydrochloride). After 30 min at room temperature, the reaction was stopped by the addition of 50 ul of 0.25 M H_2S0_4 and the A_{490} was determined using a Biorad model 3550 microplate reader. The results refer to the average values obtained from samples of 5 mice and an ELISA unit refers to the A_{490} multiplied by the appropriate dilution factor after subtraction of background values obtained from mice not immunised. Undiluted lung washes and serum samples (diluted 1 in 50) from control mice gave essentially null readings.

Electron microscopy

Uncoated and OMP-coated vesicles were negatively stained with 4 % aqueous uranyl acetate, pH 4.5, according to Valentine et al. [60]. For metal-shadowing the two vesicle samples were absorbed onto freshly prepared formvar covered nickel grids, washed with distilled water, air-dried and unidirectionally metal-shadowed with platinum (20 ° angle).

Immunoelectron microscopy

Uncoated and OMP-coated vesicles were absorbed onto freshly prepared collodium covered nickel grids and carefully washed with distilled water. After being air-dried at room temperature the grids were treated with purified antibodies (100 ug IgG protein ml^{-1}) for 30 min at room temperature. Unbound antibodies were removed by a mild spray of PBS (0.1 M potassium phosphate, 0.15 M NaCl, pH 6.9) from a plastic bottle. The bound antibodies were made visible for electron microscopy by incubating the grids on drops of protein A-gold complexes (10 nm gold particle size and A_{520} of 0.03) for 10 min at room temperature. Subsequently, the grids were rinsed with PBS containing 0.01 % Tween 20 followed by distilled water. After being air-dried the grids were unidirectionally metal-shadowed with platinum (20 ° angle). In control experiments, the samples were treated with purified preimmune immunoglobulin or with protein A-gold complexes alone. Samples were examined with a Zeiss electron microscope CEM 902 or 10B at an acceleration voltage of 80 kV and at calibrated magnifications.

RESULTS of EXPERIMENTAL PART A

Analysis of OMP-coated vesicles

The OMP-coated vesicles and the OMP preparation were analysed by SDS-PAGE (fig. 1) and found to contain essentially the same proteins indicating that the incorporation technique did not select preferentially for specfic proteins. No proteins were detected in the uncoated vesicle preparation (results not shown). Electron microscopy of the uncoated and OMP-coated vesicles revealed that both vesicles were multilamellar and varied in size from 0.2 to 2 um (fig. 2A-D). However, the OMP-coated vesicles were different in morphological appearance when compared with the uncoated vesicles; due to incorporation of OMP, the OMP vesicles exhibited blebs (fig. 2D). Incubation of such vesicles with anti-OMP antibodies resulted in an intense labeling of the vesicles (fig. 2E and F) demonstrating the location of the OMP material on the outer surface of the vesicles. In control experiments no labeling was observed (fig. 2G and H).

Immunisation with OMP-coated vesicles

To investigate the adjuvanticity of the coated vesicles, different groups of mice were vaccinated intranasally with OMP-coated vesicles and the OMP preparation used to coat the vesicles, at the same protein concentration. For mice immunised with OMP-coated vesicles, the IgA and IgG titres in the lung washes were approximately 4 fold higher than for mice immunised with the OMP preparation (fig. 3). The serum IgG titre for mice immunised by OMP-coated vesicles was about twice that for mice immunised by the OMP preparation. Mice immunised with uncoated vesicles showed no immune response to OMPs (results not shown). Although the existence of a common mucosal immune system is well established, it was of interest to compare the effectiveness of immunisation by the intranasal and oral routes. Titres in the lung washes were similar whether mice were vaccinated intranasally or orally (Table 1). A primary response was detected 10 days after the second immunisation at day 4 but titres increased substantially after a booster was given at day 30. It is not known when the peak primary response occurred but it clearly occurred after 10 days as titres were higher for mice which were killed at day 40 without having received any booster. The IgG titre in the serum paralleled that of the lung response (results not shown).

The dose required to give the maximum response in the lungs was 6 - 12 ug protein (total dose from two primary immunisations and one booster) (fig. 4). Mice immunised with 120 ug protein gave a weaker response indicating an inhibitory effect at higher concentrations. However, with doses as low as 1.25 ug, specific antibodies could still be detected in the lung washes. To determine the duration of the immune response, mice were sacrificed at various time intervals after the 30 day booster (fig. 5). The maximum IgA and IgG response both occurred 20 days after the booster but specific immunoglobulin could still be

detected in the lungs after 75 days.

Western blot analysis of the immune response

Lung washes and serum samples from mice imunised orally and intranasally were pooled together and analysed by Western blotting (fig. 6). Lung washes, from mice immunised with OMP-coated vesicles, reacted with several OMP bands although the signals were notably stronger for anti-mouse IgG than for anti IgA. This may be due to differences in avidity or sensitivity between the two detection systems or alternatively because the IgG response was more prominant than the IgA response in the lower respiratory tract. Interestingly, Nedrud et al. [36] reported that after oral and intranasal immunisation with inactivated Sendai virus, IgG was the dominant response in the lower respiratory tract whereas IgA was the dominant response in the upper respiratory tract. In control experiments, in the present study, no signals against OMP bands were detected from lung washes obtained from mice immunised with uncoated vesicles (fig. 6 lanes 3 and 4). Pooled serum from mice immunised with OMP-coated vesicles also reacted with several OMP bands (fig. 6 lane 5). Both serum and lung washes reacted with the major 32 kd OMP but the serum response showed a preference for lower molecular weight bands whereas the secretory response showed a preference for higher molecular weight bands. Serum from mice immunised with uncoated vesicles showed a very faint reaction with the major 32 kd OMP although this is cannot be seen in the photograph (fig. 6, lane 6).

Immunisation with LPS-coated vesicles

LPS isolated from B. pertussis and LPS-coated vesicles were analysed by SDS-PAGE and silver staining Both showed the same migration pattern (fig. 7) and was of the ab phenotype as described by Peppler [42]; wild type B. pertussis possesses two distinct rough LPSs and this shows itself as two bands, designated a and b, after SDS-PAGE. After oral and intranasal immunisation of mice with vesicles coated with LPS, no specific IgA could be detected in the lung washes (Table 2) although a specific IgG response was detected. However, a specific IgA response to LPS was detected when mice were immunised with the OMP-coated vesicles. Silver staining of the OMP-coated vesicles indicated the presence of LPS (results not shown) although these contained about 10 fold less LPS than LPS-coated vesicles. This suggests that proteins present in the OMP-coated vesicles are required for adequate presentation of the LPS antigens. The IgG response was also higher for mice immunised with OMP coated vesicles than for mice immunised with the LPS coated vesicles. Immunisation by the intranasal route appeared to be more effective in inducing an immune response than oral immunisation. Specific IgG to LPS was detected in the serum and the titre was similar whether OMP vesicles or LPS vesicles were used to immunise mice.

DISCUSSION OF EXPERIMENTAL PART A

Vesicles coated with OMP or LPS were shown to be effective in inducing a secretory and systemic immune response to these antigens. However, a secretory IgA response against LPS could only be detected after immunisation with the OMP-coated vesicles and not the LPS-coated vesicles. This suggests that protein antigens in the OMP preparation had had an adjuvant effect in stimulating an IgA response. Perhaps protein antigens are required for the Helper T-cell presentation that mediates B-cell isotype switching directly from IgM to IgA [54]. Mice immunised with OMP vesicles gave secretory antibody titres about four fold higher than for mice immunised with the OMP preparation. The reason for adjuvanticity of liposomes in general is not known but it is possible that the coated vesicles persist longer in the lymphoid tissue than the OMP preparation. The existence of a common mucosal immune system, where an immune response in a given mucosal tissue will result in a secretory response elsewhere, now has general acceptance [32]. However, there is some evidence that different regulatory mechanisms exist between the systems operating in gut-associated lymphoid tissue (GALT) and in the bronchus-associated lymphoid tissue (BALT) [28]. In this study, titers to OMP were similar whether mice were vaccinated orally or intranasally although mice vaccinated intranasally appeared to give a higher titer to LPS. The maximum response occurred 20 days after a 30 day booster for mice immunised intranasally with OMP-coated vesicles. However, secretory antibody could still be detected 75 days after immunisation. This is of particular interest in view of the observation of Goodman et al., [16] that anti-pertussis IgA can be detected in nasopharyngeal secretions three months after natural infection in humans, but not after parenteral vaccination. Furthermore, it is generally accepted that natural infection of pertussis results in better and longer lasting immunity than parenteral vaccination.

In this study only the humoral response after vaccination was investigated. However, other studies have shown that antigen coated liposomes can induce cell-mediated immunity (CMI) [24]. Although the role of CMI in protection against B. pertussis is not known, CMI responses to various antigens after human infection have been reported [7, 14].

The pathogenesis of pertussis is complex and it is still unclear which antigens should be included in an acellular vaccine. Parenteral vaccines based on pertussis toxin and filamentous haemagglutinin gave some protection in Swedish field trials [41]. However, it is clear that these vaccines were contaminated with other antigens which may also play a role in protection [51]. The role of anti-LPS antibodies in protection is not clear. Rabbit anti-LPS, when mixed with challenge bacteria did not protect mice from infection [47] although it has been suggested that anti-LPS antibodies in humans may still play a role in preventing colonisation [68]. The OMP-coated liposome vaccine used in this study to elicit a secretory response, is undoubtedly a crude vaccine. However, the liposome delivery system, which enhanced antibody stimulation to these antigens, could be used to incorporate purified surface antigens such as

filamentous haemagglutinin, pertussis toxin, or the 69 kd OMP associated with virulence, which have been shown to be protective in mouse models [67]. The soya bean lipid source, used to manufacture these liposomes is relatively cheap and therefore attractive for large-scale vaccine production.

On the other hand, complete protection against B. pertussis may require an immune response to several antigens and it is perhaps false dogma that future vaccines should only consist of defined purified components. The oral route of administration may circumvent many of the toxic side affects associated with parenteral administration of crude vaccines especially those associated with LPS. Side effects associated with pertussis toxin would also certainly be expected to be reduced. However, it may be desirable either to toxoid the preparation before liposome incorporation or to use the recently described B. pertussis mutant which expresses an immunologically active but biologically inactive pertussis toxin [37]. It is of interest to note that in a study of 15,000 infants, a whole-cell vaccine given orally was equally as effective as vaccine given parenterally but side reactions were essentially eliminated with oral immunisation [9]. However, the length of immunity conferred by oral immunisation was not further investigated.

In conclusion, this study has demonstrated the usefulness of the liposome delivery system to enhance stimulation of secretory antibodies to B. pertussis surface antigens. Further studies, however, are required to determine whether these protect mice from colonisation and whether the liposomes can be used as a delivery system for individual B. pertussis surface antigens.

EXPERIMENTAL PART B: THERAPEUTIC ASPECTS

MATERIALS AND METHODS

Preparation of antigen-entrapped vesicles. FHA was purified from B. pertussis Tohama strain as previously described by Sato et al. [50] and PT was kindly supplied by S. Cryz and detoxified as described by Munoz et al. [35]. Lyphazome ^{TM} liposomes, a proprietary form of small multilamelar phospholipid vesicles, containing B. pertussis FHA and PT were prepared using the solvent dilution microcarrier technique developed by Fountain Pharmaceuticals Inc. [53], using purified soya bean phosphatides supplied by American Lecithin Company, New York. The composition of the purified lipid mixture was phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid and neutral lipids in an approximate ratio of 8:1:0.7:0.3.

Mice immunization. Five to six week old female BALB/c mice (Charles River, MI, Italy) were orally immunized in groups of five and caged separately according to the following protocol: group a,

liposome-entrapped FHA; group b, liposome-entrapped PT; group c, liposome-entrapped FHA and liposome-entrapped PT; group d, free FHA; group e, free PT; group f, free FHA and PT; a control group was immunized using uncoated liposomes. The vaccination regime was one dose of protein (4 ug) on day 0 and 4, followed by a booster of an identical dose on day 30. Mice that had been deprived of water for 6 - 8 h were gently fed with 50 ul of vaccine diluted in PBS and an equal volume of 3 % sodium bicarbonate in phosphate-buffered saline (PBS: NaCl [8.0 g liter^{-1}], KCl [2.0 g liter^{-1}], $Na_2HPO_4.2H_2O$ [2.0 g liter^{-1}], KH_2PO_4 [2.0 g liter^{-1}) (pH 8.0), which was added immediately before to neutralize gastric acidity. The animals were sacrificed 10 days after the booster and the samples were collected. Sacrificed mice were exsanguinated by cutting the brachial artery, and the collected serum was separated and stored at -20 °C. Lung washes were collected by pertracheal cannulation and gentle washing with 0.7 ml of ice cold PBS containing 2 mM phenyl-methane-sulfonylfluoride as protease inhibitor. About 0.5 ml of lung wash was recovered from each mouse, centrifuged at 4 °C at 10,000 x g for 5 min to remove debris and stored at -20 °C.

Immunological techniques. Monoclonal antibody P12H3 against FHA [13] and a cocktail of monoclonal antibodies reactive against PT subunits S1 (E19), S4/S5 doublet (E205), and S2 and S3 (E251) [65] were used in western blotting experiments [5]. Free and liposome-entrapped FHA and PT were mixed with loading buffer in a ratio of 1:1 and protein was electrophoresed according to the procedure of Laemmli [30] using a 3.85 % acrylamide stacking gel and a 10 % acrylamide separating gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad Labs. S.r.l., Mi, Italy) with a semi-dry transfer cell (Bio-Rad) using 25 mM Tris, 192 mM glycine, 20 % methanol pH 8.3 as the transfer buffer and a 10 % solution of 0.3 % fat milk in PBS pH 7.4 as the blocking reagent. The blocked membrane was incubated for 2 h with the first antibody, either P12H3 and a cocktail of E19, E205, and E251 hybridoma supernatant fluids diluted 1:20 in PBS (pH 7.4). After three washes with PBS the membranes were incubated for 1 h with horse radish peroxidase conjugated goat anti-mouse IgG diluted 1:500 in PBS (Southern Biotechnology Associates, Inc., Birmingham, Ala.). Membranes were washed and developed using 4chloro-1-naphthol as substrate. Prestained molecular weight markers were purchased from Bio-Rad.

For the determination of subclass specific antibodies against FHA and PT present in serum and lung washes, enzyme-linked immunosorbent assays (ELISA) were performed as follows. Nunc Maxisorp Immunomodule 96-well plates were coated with FHA or PT diluted in 0.1 M $NaHCO_3$ (pH 9.6, 60 ng in 50 ul per well, incubated at 4 °C overnight). The wells were blocked with 100 ul of 10 % fetal calf serum (FCS, Flow Laboratories Inc., Irvine, United Kingdom) in PBS for 2 h at 37 °C. Plates were subsequently washed three times with PBS, and 100 ul of serum samples diluted 1:50 or lung washes diluted 1:10 in 10 % FCS in PBS were added to each well. After 60 min at 37 °C the plates were again washed and 100 ul of alkaline phosphatase-conjugated goat anti-mouse antibodies for IgG, IgM, or IgA heavy chains

(Southern Biotechnology Associates, Inc.) diluted 1:500 in 10 % FCS in PBS were added to each well and incubated for 2 h at 37 °C. The plates were again washed and then developed by the addition of 100 ul per well of the substrate solution (10 mg/ml pnitrophenylphosphate, disodium salt, in diethanolamine buffer pH 9.8). After 30 min at room temperature, the reaction was stopped by the addition of 50 ul of 3.0 M NaOH and the A_{405} was determined with a Titretek Multiskan MCC microplate reader (Flow). All samples were processed simultaneously on the same day, each serum or lung wash sample was individually assayed and antigen-free liposome-immunized mouse serum or lung washes were used as the blank for the ELISA readings. Results are expressed as mean values of each immunization group; standard deviations represent variations between individual mouse samples in each group.

Electron Microscopy. Uncoated, FHA-coated and PT-coated liposomes were negatively stained with 4 % aqueous uranyl acetate pH 4.5, according to Valentine et al. [60]. For metal-shadowing, the liposome samples were absorbed onto freshly prepared collodium covered 300 mesh nickel grids, washed with distilled water, air-dried and unidirectionally metal-shadowed with platinum at an angle of 15 °

For immunoelectron microscopy, uncoated or antigen-coated liposomes were absorbed onto freshly prepared collodium covered 300 mesh nickel grids and carefully washed with distilled water. After being air-dried at room temperature the grids were treated with protein A purified anti-FHA or PT polyclonal or monoclonal antibodies (1.25 ug IgG protein ml^{-1}) for 60 min at room temperature. Unbound antibodies were removed by a mild spray of PBS (pH 6.9) from a plastic bottle. The bound antibodies were made visible for electron microscopy by incubating the grids on drops of protein A-gold complexes (10 nm gold particle size, A_{520} of 0.01) for 15 min at room temperature. Subsequently, the grids were rinsed with PBS containing 0.01 % Tween 20 followed by distilled water. After being air-dried the grids were unidirectionally metal-shadowed with platinum (15 ° angle) or examined without metal-shadowing. In control experiments, the samples were either treated with purified preimmune serum or with protein A-gold complexes alone. For post-embedding labeling the pertussis toxin liposomes were embedded according to the progressive lowering of temperature method using Lowicryl K4M resin and applying the labeling protocol as described [64]. Samples were examined with a Zeiss electron microscope EM 10B at an acceleration voltage of 80 kV and at calibrated magnifications.

RESULTS OF EXPERIMENTAL PART B

Size determination of liposomes containing FHA and PT. Size analysis studies of the FHA and PT coated liposomes were performed using a Coulter Counter model N4M PT. The mean diameter of blank, and FHA or PT coated liposomes were 227 nm (95 % limits 211 to 243 nm), 236 nm (95 % limits 219 to

253 nm), and 244 (95 % limits 226 to 262 nm), respectively. These data demonstrate that the entrapment of FHA and PT into the liposomes does not significantly affect their average diameter.

Western blot analysis of liposomes containing FHA and PT. The presence of FHA and PT in the vesicles was confirmed by western blot analysis (Fig. 8) using P12H3 monoclonal antibody (lanes 2, 3 and 4) and the cocktail of hybridoma supernatants against PT subunits S 1-4 (lanes 2, 5 and 6). No differences in FHA content were detected between free FHA and FHA-coated liposomes (lanes 3 and 4). On the other hand, differences between free PT and liposome-incorporated PT were evident (lanes 5 and 6), suggesting either variations in binding affinity between monoclonal antibodies specific for S1/S4, and S2/S3 or preferential incorporation of S1 and S4/5 subunits in the liposomes. No proteins were detected into the blank-uncoated liposomes (lane 2).

Immuno-electron microscopic analysis of liposomes containing FHA and PT. Electron microscopy of the uncoated, FHA-coated, and PT-coated liposomes revealed that the liposomes were multilamellar and varied in size (Fig. 9. A, B, C and Fig. 3. A). However, the FHA-coated and PT-coated liposomes exhibit a different morphological appearance when compared with the uncoated liposomes (compare Fig.9 A and B, Fig.9 A and Fig.10 A). Most probably due to the incorporation of FHA and PT into the liposomes. Incubation of the FHA-coated liposomes with anti-FHA polyclonal or monoclonal antibodies followed by protein A-gold complexes resulted in an intensive labeling pattern on the surface of the FHA-liposomes (Fig. 9 D and E). Incubation with monoclonal antibodies revealed less labeling (data not shown). Incubation of the PT-coated liposomes with polyclonal anti-PT antibodies resulted in a much weaker labeling pattern than for the FHA-coated liposomes (compare Fig. 9. D and Fig. 10. B). Since the label intensity was sometimes only slightly above the background label we have undertaken to demonstrate by post-embedding labeling that PT can be detected on the liposome membrane. The former method allows only for the detection of antigenic reactive sites which stick out of the liposomes but not for those which reside on the inner side of the liposome membrane. In Fig. 10. D a liposome is depicted which exhibit a label around the membrane demonstrating that PT is incorporated in the external membrane. In control experiments (Fig. 9. F and Fig. 10. C) only a very few gold-particles could be detected.

Antibody responses specific for B. pertussis FHA and PT in vaccinated mice. Both free FHA and PT, and PT and FHA antigen-entrapped liposome prototype vaccines, elicited specific serum and mucosal antibody responses (Fig. 10 and 11). After oral immunization of mice with a total dose of 12 ug of FHA or PT administrated in three equal doses, both systemic (mainly IgG) and lung secretory (mainly IgG and IgA) antibody responses were obtained (Fig. 10 A and B). The adjuvanticity of the liposome-based system was confirmed for FHA by the presence of antibodies titres approximately three times higher in mice immunized with FHA-entrapped liposomes than in those immunized orally with free protein (Fig.

10). Simultaneous vaccination with FHA and PT, both free and liposome entrapped, did not affect anti-FHA antibody responses. The titres of FHA specific IgM detected in lung washes were not significant (data not shown).

Similar results were obtained by immunization with PT (Fig. 11 A and B) and as with FHA, co-immunization with both proteins (FHA and PT) gave good anti-PT antibody responses.

DISCUSSION OF EXPERIMENTAL PART B

Current parenteral vaccines against whooping cough are more efficient in protecting against clinical pertussis than against infection. In designing new, safer vaccines however, it would seem appropriate to reconsider the immunization strategy and to take into account respiratory tract portal of entry of pertussis infection and the possibility of vaccines that stimulate efficient responses at mucosal level which block infection. Secretory IgA and specific IgG (derived from serum by transudation through capillary vessels and being the predominant Ig in the lower respiratory tract) may interfere with the early events of bacterial attachment and colonization and thereby facilitating the eradication of the disease.

The processing of antigens administered by oral and parenteral routes differs regarding antigenic specificity and Ig class profile [2, 6, 27, 58], IgA responses being typical of oral vaccination. The specialized M cells which cover the Peyer's patches pass antigenic material to lymphocytes below the epithelium, where the processed antigens are presented to IgA-precursor B cells. These B cells travel via the lymphatic system to the different mucosae and then give rise to IgA secreting plasma cells. T lymphocytes may also acquire their homing pattern in the Peyer's patches [58].

For a number of antigens, mucosal and systemic immune responses have been obtained by oral administration [23, 26, 56, 61] which protect and persist longer than those induced by antigen administrated via the parenteral route. B. pertussis whole cell vaccine given orally was demonstrated to be as effective as parenterally administered vaccine [9]. We have recently demonstrated specific lung mucosal responses against FHA and the S1 subunit of PT after oral immunization with recombinant aro A mutans of Salmonella spp. [21, 63]. The present work extends these observations by demonstrating that systemic and mucosal immune responses against B. pertussis antigens are obtained following oral immunization with free FHA and PT. The encapsidation of FHA and PT into the liposome delivery system resulted in an adjuvant effect with greatly improved antibody responses. Tolerance and systemic suppression as a result of antigen-specific T suppressor cells and absorption problems due to preexisting levels of specific secretory IgA have been described after administration of antigens by oral route [6, 58]. However, these phenomena were not seen in our experiments.

It is important to highlight that in mice immunized with both FHA-coated and PT-coated liposomes, the antibody levels obtained were the same in those immunized with a single antigen. We conclude therefore that this approach can be used to deliver multiple antigens including the 69 kDa outer membrane protein of B. pertussis which was recently demonstrated to be an important protective antigen [39]. E. coli strains which express recombinant FHA [20], Bordetella spp. strains which overproduce PT [64, 69] and methods for the genetic detoxification of PT [44, 69] are now available and will facilitate the production of high yields of non-toxic, full-antigenic components for liposome based subunit vaccines. In fact, multivalent vaccines can be envisaged which could replace the old DTP vaccine in infant vaccination regimes, as enhancement of the primary and secondary immune responses has resulted from diphtheria toxoid association with liposomes [1].

The mechanisms which mediate protection to B. pertussis infection are not clearly understood. Humoral and cellular responses following parenteral immunization do not always correlate [44]. Moreover, cell-mediated immune responses are believed to be important in vivo for protection against whooping cough disease [7, 14]. As liposomes are known to induce cell-mediated immunity, it will be of interest to determine whether the delivery systems used in this study induce cell-mediated responses to FHA and PT in addition to the documented antibody responses.

The results reported here constitute only a starting point: the incorporation of other products known to have adjuvant activity, including lipid A and aluminum hydroxide, may have additive or synergistic effects on secretory IgA priming [43, 46]; administration of vitamin A may improve the immune responses after oral vaccination [2]; and other aspects, including the length of the immunity obtained, optimal vaccination schedule, and protection after challenge must be also investigated.

The use of liposomes as delivery system for subunit vaccines to induce immune responses distal to the site of entry are very promising. The oral route may reduce or eliminate the most frequent local side effects associated with whooping cough vaccines, then adjuvanticity may increase both cell mediated and humoral immune responses. Liposomes are chemically stable, simple to manufacture and biodegradable; toxicity was not reported after using liposomes in phase I and II trials [1, 17]. Moreover, the inexpensive raw materials used to produce the liposomes will contribute to reduced production costs. The oral administration of vaccines is in any case associated with a major cost-saving vis-à-vis parenteral vaccination and is a key aspect of major vaccination programs, particularly in rural settings where the health care delivery is very expensive.

TABLE 1. Antibody titres to OMP of B. pertussis in mouse lung washes after oral and intranasal administration of OMP-coated vesicles.

Route of administration	10 days[a]		40 days[a]			
			Without booster[b]		With booster	
	IgA	IgG	IgA	IgG	IgA	IgG
Oral	0.10±0.06[c]	0.11±0.05	nd[d]	nd	4.6±2.0	6.5±1.1
Intranasal	0.11±0.03	0.35±0.17	0.40±0.18	0.48±0.35	5.1±1.5	6.6±1.5

a, days after first immunisation; b, a booster was given 30 days after the first immunisation; c, standard deviation nd = not determined.

TABLE 2. Antibody titres to LPS of B. pertussis in mouse lung washes after oral and intranasal administration with LPS- and OMP-coated vesicles.

	Oral		Intranasal	
	IgA	IgG	IgA	IgG
LPS-vesicles	0.0±0.05[a]	0.37±0.12	0.0±0.05	0.46±0.21
OMP-vesicles	0.66±0.51	1.1±0.34	1.6±0.23	1.6±0.14

a, standard deviation.

[1] **Allison A. C. and G. Gregoriadis.** 1974. Liposomes as immunological adjuvants. Nature 252.252.

[2] **Bienenstock J. and A. D. Befus.** 1980. Mucosal immunology. 41:249-270.

[3] **Brownlie R. M., J. G. Coote and R. Parton.** (1985) Adenylate cyclase activity activity during phenotypic variation of Bordetella pertussis. J. Gen. Microbiol. 131, 27-38.

[4] **Bulow R. and J. C. Boothroyd.** 1991. Protection of mice from fatal Toxoplasma gondii infection by immunization with p30 antigen in liposomes. J. Immunol. 147:3496-3500.

[5] **Burnette W. N.** 1981 "Western blotting" electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to umnodified nitrocellulose and radiografic detection with antibody and radioiodinated protein A. Analyt. Biochem. 112:195-203.

[6] **Czerkinsky C., S. J. Prince, S. M. Michalek, S. Jackson, M. W. Russell, Z. Moldoveanu, J. R. McGhee and J. Mestecky.** 1987. IgA antibody-producing cells in peripheral blood aRer antigen ingestion: evidence for a common mucosal immune system in humans. Proc. Natl. Acad. Sci. USA. 84:2449-2453.

[7] **De Magistris T. M., M. Romano, S. Nuti, R. Rappuoli and A. Tagliabue.** 1988. Dissecting human T cell responses against Bordetella species. J. Exp. Med. 168:1351-1362.

[8] **Desiderio J. V. and S. G. Campbell.** 1985. Immunization against experimental murine Salmonellosis with liposome-associated O-antigen. Infect. Immun. 48:658-663.

[9] **Falk W., K. H. Hofler, K. Rosanelli and R. Kurz.** 1981. Gegenwart and Zukunft der Oralen Pertussis Schutzimpfung. Fortschr. Med. 99:1363-1366.

[10] **Francis M. J., C. M. Fry, D. 1. Rowlands, F. Brown, J. L. Bittle, R. A. Houghten and R. A. Lerner.** 1985. Immunological priming with synthetic peptides of foot-and-mouth disease virus. J. Gen. Virol. 66:2347-2354.

[11] **Fine P. E. M. and J. A. Clarkson** (1982) The recurrence of whooping cough: possible implications for assessment of vaccine efficacy. Lancet I, 666-669.

[12] **Fine P. E. M. and J. A. Clarkson.** 1987. Reflections in the efficacy of pertussis vaccines. Rev. Infect. Dis. 9:866-883.

[13] **Frank D. W. and C. D. Parker.** 1984. Isolation and characterization of monoclonal antibodies to Bordetella pertussis. J. Biol. Stand. 12:353-365.

[14] **Gearing A. J. H., C. R. Bird, K. Reedhead and M. Thomas.** 1989. Human cellular immune responses to Bordetella pertussis infection. FEMS. Microbiol. Immunol. 47:205-212.

[15] **Giammanco A., A. Chiarini, T. Stroffolini, D. De Mattia, M. Chiararnonte, M. E. Moschen, I. Mura, G. Rigo, S. Taormina, A. Sarzana, G. Mazza and B. Scarpa.** 1991. Seroepidemiology of Pertussis in Italy. Rev. Infect. Dis. 13:1216-1220.

[16] **Goodman Y. E., A. J. Wort and F. L. Jackson** (1981) Enzyme linked immunosorbent assay for detection of pertussis immunoglobulin A in nasopharyngeal secretions as indicator of recent infection. J. Clin. Microbiol. 13, 286-292.

[17] **Gregoriadis G.** 1990. Immunological adjuvants: a role for liposomes. Immunology Today. 11.89-97

[18] **Gregory R. L., S. M. Michalek, G. Richardson, C. Harmon, T. Hilton and J. R. McGhee.** 1986 Characterization of immune response to oral administration of Streptococcus sobrinus ribosomal preparations in liposomes. Infect. Immun. 54:780-786.

[19] **Griffiths E.** (1988) Efficacy of whole-cell pertussis vaccine. In: Pathogenesis and Immunity in Pertussis (Eds. A.C. Wardlaw and R. Parton) pp 353-374. John Wiley and Sons Ltd. Guildford, U.K.

[20] **Guzmán C. A., M. J. Walker, M. Rohde and K. N. Timmis.** 1991. Direct expression of Bordetella pertussis filamentous hemagglutinin in Escherichia coli and Salmonella typhimurium aroA. Infect. Immun. 59:3787-3795 .

[21] **Guzmán C. A., R. M. Brownlie, J. Kadurugamuwa, M. J. Walker and K. N. Timmis.** 1991 Antibody responses in the lungs of mice following oral immunization with Salmonella typhimurium aro A and invasive Escherichia coli strains expressing the filamentous hemagglutinin of Bordetella pertussis. Infect. Immun. 59:4391-4397.

[22] **Howson C. P. and H. V. Fineberg.** 1992. Adverse events following Pertussis and Rubella vaccines: summary of a report of the institute of medicine. JAMA 267:392-396.

[23] **Johansen H., N. Holby and S. S. Pedersen.** 1991. Experimental immunistion with Pseudomonas aeruginosa alginate induces IgA and IgG antibody responses. APMIS. 99:1061-1068.

[24] **Kahl L. P., R. Lelchuk, C. A. Scott and J. Beesley.** 1990. Characterlstion of Leishmania major antigen-liposomes that protect BALB/c mice against cutaneous leishmaniasis. 58:3233-3241.

[25] **Kahl L. P., C. A. Scott, R. Lelchuk, G. Gregoriadis and F. Y. Liew.** 1989. Vaccination against murine cutaneous leishmaniasis by using Leishmania major antigen/liposomes. J. Immunol 142 4441-4449.

[26] **Kantele A., H. Arvilommi and I. Jokinen.** 1986. Specific immunoglobulin-secreting human blood cells after peroral vaccination against Salmonella typhi. J. Infect. Dis. 153:1126-1131.

[27] **Kantele A., H. Arvilommi, J. M. Kantele, L. Rintala and P. H. Makela.** 1991. Comparison of the human immune response to live, killed oral, or killed parenteral Salmonella typhi Ty21a vaccines. Microbial. Pathogen. 10:117-126.

[28] **Kett K., P. Brandtzaeg, J. Radl and J.F. Haaijman** (1986) Different subclass distribution of IgA-producing cells in human lymphoid organs and various secretory tissues. J. Immunol. 136, 3631-3635.

[29] **Kimura A., K. T. Mountzouros, D. A. Relman, S. Falkow and J. L. Cowell.** 1990. Bordetella pertussis filamentous hemagglutinin: evaluation as a protective antigen and colonization factor in a mouse respiratory infection model. Infect. Immun. 58:7-16

[30] **Laemmli U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

[31] **Larson E., Howlett B. and Jagendorf A.** (1986) Artificial reductant enhancement of the Lowry method for protein determination. Anal. Biochem. 155, 243-248.

[32] **Mestecky J.** (1987) The common mucosal immune system and current strategies for induction of immune responses in external secretions. J. Clin. Immunol. 7, 265-276.

[33] **Miller D. L., R. Alderslade and E. M. Ross.** 1982. Whooping cough and whooping cough vaccine: the risks and benefits debate. Epidemiol. Rev. 4:1-24.

[34] **Muller A. S., J. Leeuwenburg and D. S. Pratt.** 1986. Pertussis: epidemiology and control. Bull WHO. 64:321-33 1.

[35] **Munoz J. J., H. Arai and R. L. Cole.** 1981. Mouse-protecting and histamine-sensitizing activities of pertussigen and fimbrial hemagglutinin from Bordetella pertussis. Infect. Immun. 32:243-250.

[36] **Nedrad J.G., Liang X.-P. and Lamm M.E.** (1989) Oral immunization with Sendai virus in mice. Curr. Topic. Microbiol. 146, 117-122.

[37] **Nencioni L., Pizza M., Bugnoli M., De Magistris T., Di Tommaso A., Giovannoni F., Manetti R., Marsili I., Matteucci G., Nucci D., Olivieri R., Pileri P., Presentini R., Villa L., Kreeftenberg J.G., Silvestri S., Tagliabue A. and Rapouli R.** (1990) Characterisation of genetically inactivated pertussis toxin mutants: candidates for a new vaccine against whooping cough. Infect. Immun. 58, 1308-1315.

[38] **Neurath R. A., S. B. Kent and N. Strick.** 1984. Location and chemical synthesis of a Pre-S gene coded immunodominant epitope of hepatitis B virus. Science 224:392-394.

[39] **Novotny P., A. P. Chubb, K. Cownley and I. G. Charles.** 1991. Biologic and protective properties of the 69 kDa outer membrane protein of Bordetella pertussis: a novel formulation for an acellular pertussis vaccine. J. Infect. Dis. 164:114-122.

[40] **Ölander R. M., A. Muotiala, M. Karvonen, T. Kuronen and K. Runeberg-Nyman.** 1990. Serum antibody response to B. pertussis Tn5 mutants, purified PT and FHA in two different mouse strains and passive protection in the murine intranasal infection model. Microbial. Pathogen. 8:37-45.

[41] **Olin P. and Storsaeter J.** (1989) The efficiency of acellular pertussis vaccine. J. Amer. Med. Assoc. 261, 560-562.

[42] **Peppler M.S.** (1984) Two physically and serologically distinct lipopolysaccharide profiles in strains of Bordetella pertussis and their phenotype variants. Infect. Immun. 43, 224-232.

[43] **Pierce N. F. and J. B. Sacci Jr.** 1984. Enhancement by lipid A of mucosal immunogenicity of liposome-associated cholera toxin. Rev. Infect. Dis. 6:563-566.

[44] **Podda A., L. Nencioni, T. M. De Magistris, A. Di Tommaso, P. Bossù, S. Nuti, P. Pileri, S. Peppoloni, M. Bugnoli, P. Ruggiero, L Marsili, A. D'Errico, A. Tagliabue and R. Rappuoli.** 1990. Metabolic humoral and cellular responses in adult volunteers immunized with the genetically inactivated pertussis toxin mutant PT-9K/129G. J Exp. Med. 172:861-868.

[45] **Rhalem A., C. Bourdieu, G. Luffau and P. Pery.** 1988. Vaccination of mice with liposome-entrapped adult antigens of Nippostrongylus brasiliensis. Ann. Inst. Pasteur/Immunol 139:157-166.

[46] **Richards R. A., M. D. Hayre, W. T. Hockmeyer and C. R. Alving.** 1988. Liposomes, lipid A, and aluminum hydroxide enhance the immune response to a synthetic Malaria sporozoite antigen. Infect. Immun. 56:682-686.

[47] **Robertson A., Ashworth L.A.E., Baskerville A. and Irons L.I.** (1985) Protection against intranasal infection of mice with Bordetella pertussis. Develop. Biol. Standard. 61, 165-172.

[48] **Robertson A., Irons L.I. and Ashworth L.A.E.** (1985) Pertussis vaccine: present status and future prospects. Vaccine 3, 11-22.

[49] **Russel D. G. and J. Alexander.** 1988. Effective immunization against cutaneous leishmaniasis with defined membrane antigens reconstituted into liposomes. J. Immunol. 140:1174-1279.

[50] **Sato Y., Cowell J. L., Sato H., Burstyn D. G. and Manclark C. R.** 1983. Separation and purification of the hemagglutinins from Bordetellu pertussis. Infect. Immun. 41:313-320.

[51] **Shahin R.D., Brennan M.J., Li Z.M., Meade B.D. and Manclark C.R.** (1990) Characterization of the protective capacity and immunogenicity of the 69 kd outer membrane protein of Bordetella pertussis J. Exp. Med. 171, 63-73.

[52] **Snippe H., J. E. G. van Dam, A. J. van Houte, J. M. N. Willers, J. P. Kamerling and J. F. G. Vliegenthart.** 1983. Preparation of a semisynthetic vaccine to Streptococcus pneumoniae type 3. Infect. Immun. 42:842-844.

[53] **Spanish patent number 2013531.** 1990. Method for making solvent dilution microcarriers. March 30 1990.

[54] **Strober W. and Sneller M.C.** (1988) Cellular and molecular events accompanying IgA B cell differentiation. Mono. Allergy 24, 181-190.

[55] **Sutter R. W. and Cochi S. L.** 1992. Pertussis hospitalizations and mortality in the United States 1985-1988. Evaluation of the completeness of national reporting. JAMA 267:386-391.

[56] **Svennerholm A. M., Sack D. A., Holmgren J. and Bardhan P. K.** 1982. Intestinal antibody responses after immunization with cholera B subunit. Lancet 305-308.

[57] **Thomas M. G., E. L. A. Ashworth, E. Miller and H. P. Lambert.** 1989. Serum IgG, IgA, and IgM responses to pertussis toxin, filamentous hemagglutinin, and agglutinogens 2 and 3 after infection with Bordetella pertussis and immunization with whole-cell vaccine. J. Infect. Dis. 160:838-845.

[58] **Tomasi Jr. T. B.** 1983. Mechanisms of immune regulation at mucosal surfaces. Rev. Infect. Dis. 5(suppl):S784-S792.

[59] **Tsai C.M. and Frasch C.E.** (1982). A sensitive silver stain for detecting lipopolysaccharides in polysaccharides in polyacrylamide gels. Anal. Biochem. 119, 115-119.

[60] **Valentine R. C., B. M. Shapiro and E. R. Stadtman.** 1968. Regulation of glutamine synthetase XII. Electron microscopy of the enzyme from Escherichia coli. Biochem. 7:2143-2152.

[61] **Wachsmann D., J. P. Klein, M. Scholler, J. Ogier, F. Ackermans and R. M. Frank.** 1986. Serum and salivary antibody responses in rats orally immunized with Streptococcus mutants carbohydrate protein conjugate associated with liposomes. Infect. Immun. 52:408-413.

[62] **Walker E.** Clinical aspects of pertussis, in A. C. Wardlaw and R. Parton (eds). Pathogenesis and Immunity in Pertussis. John Wiley and Sons, Chichester. pp. 273-282.

[63] **Walker M. J., M. Rohde, K. N. Timmis and C. A. Guzmàn.** 1992. Specific lung mucosal and systemic immune responses after oral immunization of mice with Salmonella typhimurium aroA, Salmonella typhi Ty 21a and invasive Escherichia coli expressing recombinant pertussis toxin subunit. Submitted to Infect. Immun.

[64] **Walker M. J., M. Rohde, J. Wehland and K. N. Timmis.** 1991. Construction of minitransposons for constitutive and inducible expression of pertussis toxin in bvg-negative Bordetella bronchiseptica. Infect Immun. 59:4238-4248.

[65] **Walker M. J., J. Wehland, K. N. Timmis, B. Raupach and M. A. Schmidt.** 1991. Characterization of murine monoclonal antibodies that recognize defined epitopes of pertussis toxin and neutralize its toxin effect on Chinese Hamster ovary cells. Infect. Immun. 59:4249-4251.

[66] **Wardlaw A.C. Parton R. and Hooker M.J.** (1976) Loss of protective antigen, histamine-sensitizing factor and envelope polypeptides in cultural variants of Bordetella pertussis. J. Med. Microbiol. 9, 89-100.

[67] **Weiss A.A. and Hewlett E.L.** (1986) Virulence factors of Bordetella pertussis. Ann. Rev. Microbiol. 40, 661-686.

[68] **Winsnes R. Lonnes T. Mogsten B. and Bendal B.P.** (1985) Antibody responses after vaccination and disease against leukocytosis promoting factor, filamentous hemagglutinin lipopolysaccharide and a protein binding to complement-fixing antibodies induced during whooping cough. Develop. Biol Standard. 61, 353-365.

[69] **Zealey G. R., S. M. Loosmore, R. K. Yacoob, S. A. Cockle, A. B. Herbert, L. D. Miller, N. J. Mackay and M. H. Nein.** 1992. Construction of Bordetella pertussis strains that overproduce genetically inactivated pertussis toxin. Appl. Env. Microbiol. 58:208-214.

CLAIMS

1. Vaccine against pathogens of mucosae where the vaccine consists of a liposome delivery system or contains said delivery system as reactive component where the delivery system consists of lipid vesicles or contains same characterized in that the lipid vesicles contain at least one antigen of the pathogens of mucosae incorporated into their outer membrane.

2. Vaccine according to claim 1 characterized in that the pathogen of mucosae is a strain of a species of Bordetella, especially B, pertussis, shigella or streptococcus.

3. Vaccine according to claim 2, characterized in that the antigen is a preparation of the outer membrane protein (OMP) of Bordetella pertussis.

4. Vaccine according to claim 2, characterized in that the antigen is a lipopolysaccharide (LPS) of Bordetella pertussis.

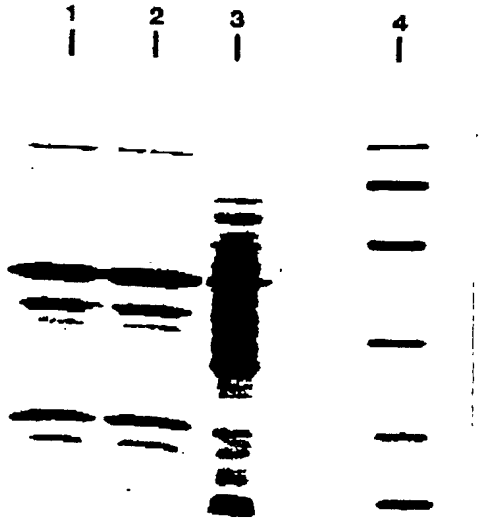
5. Vaccine according to claim 2, characterized in that the antigen is filamentous haemaglutinin and/or pertussis-toxin of Bordetella pertussis.

6. Vaccine according to claim 2, characterized in that both a preparation of the outer membrane protein (OMP) and a lipopolysaccharide (LPS) as well of Bordetella pertussis are contained as antigen.

7. Vaccine according to claim 1, characterized in that the vaccine according to lipid-vesicles are small multilamelar phospholipid-vesicles based on soybean.

8. Vaccine according to any of the preceding claims, characterized in that it is applied for oral application for immunizing mucosae, especially for immunizing intestinal and/or breathing passages, preferably for immunizing breathing passages.

9. Process for the preparation of a vaccine according to any of the preceding claims, characterized in that the antigen or the antigens are incorporated by means of the solvent dilution microcarrier technique into the lipid-vesicles.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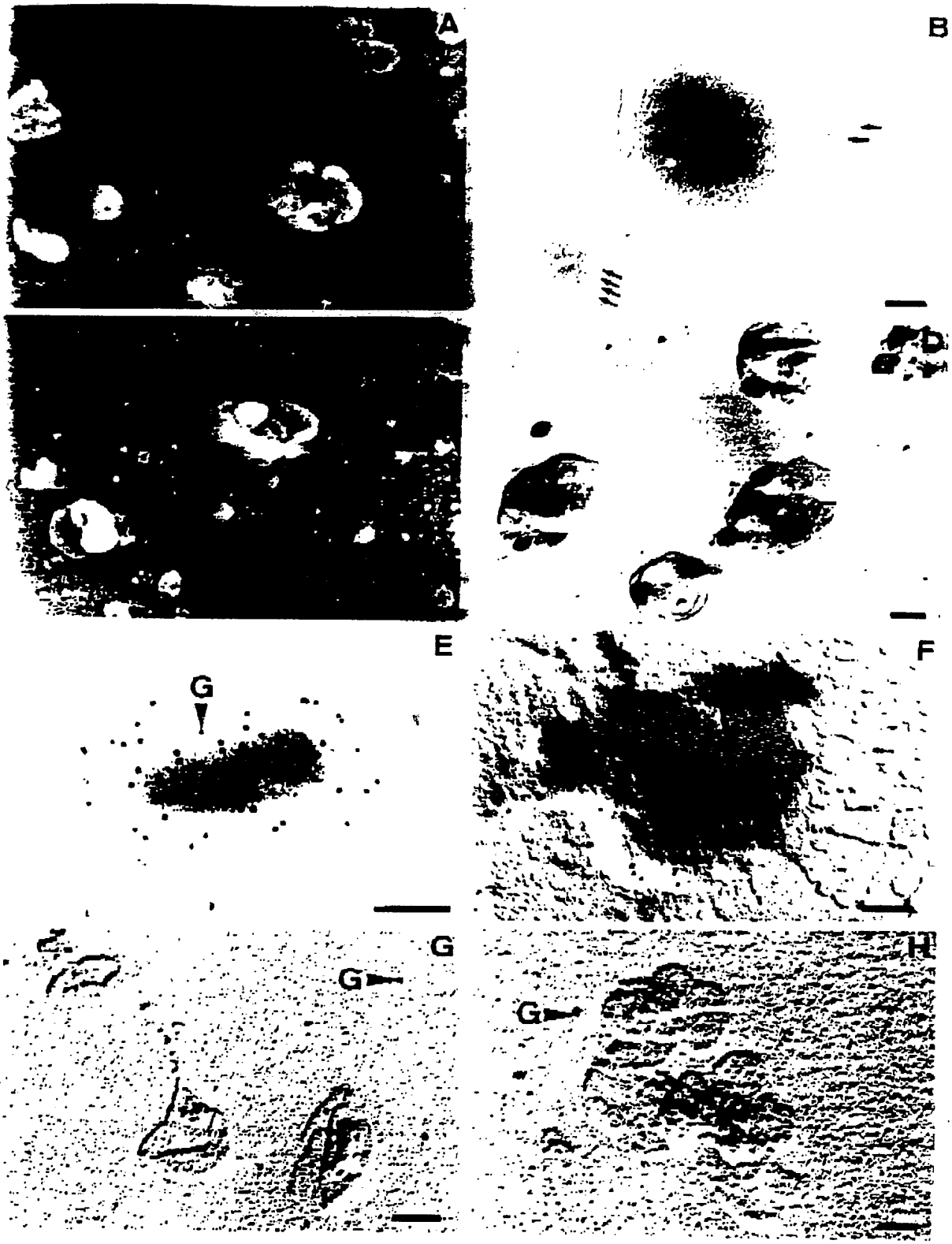
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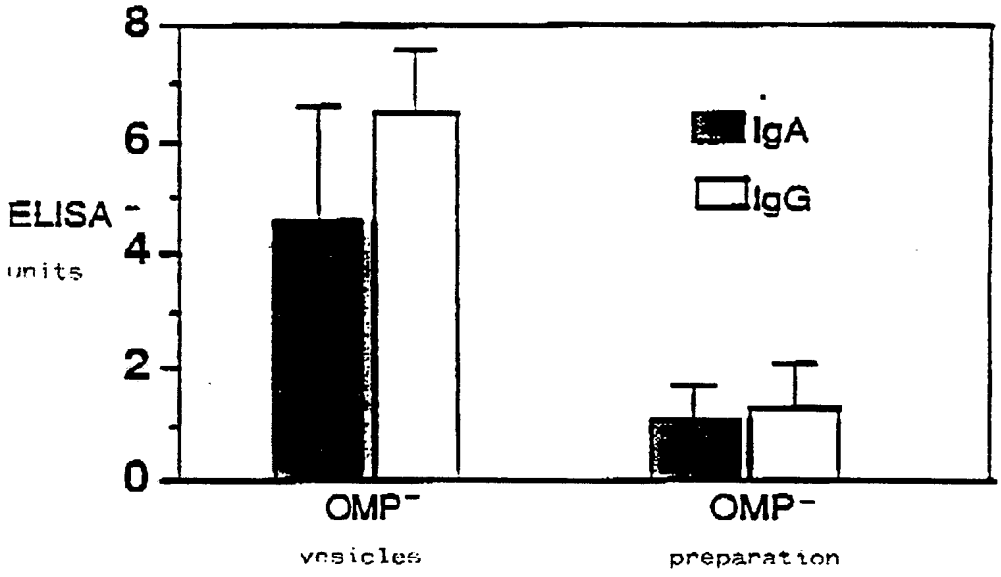
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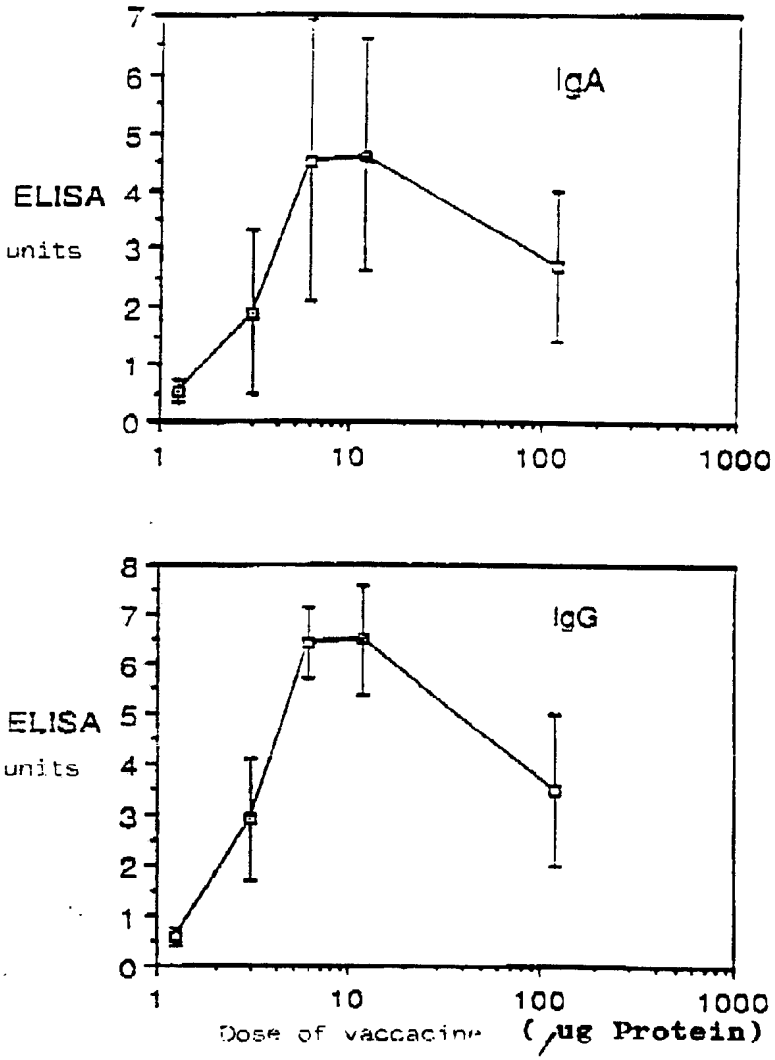
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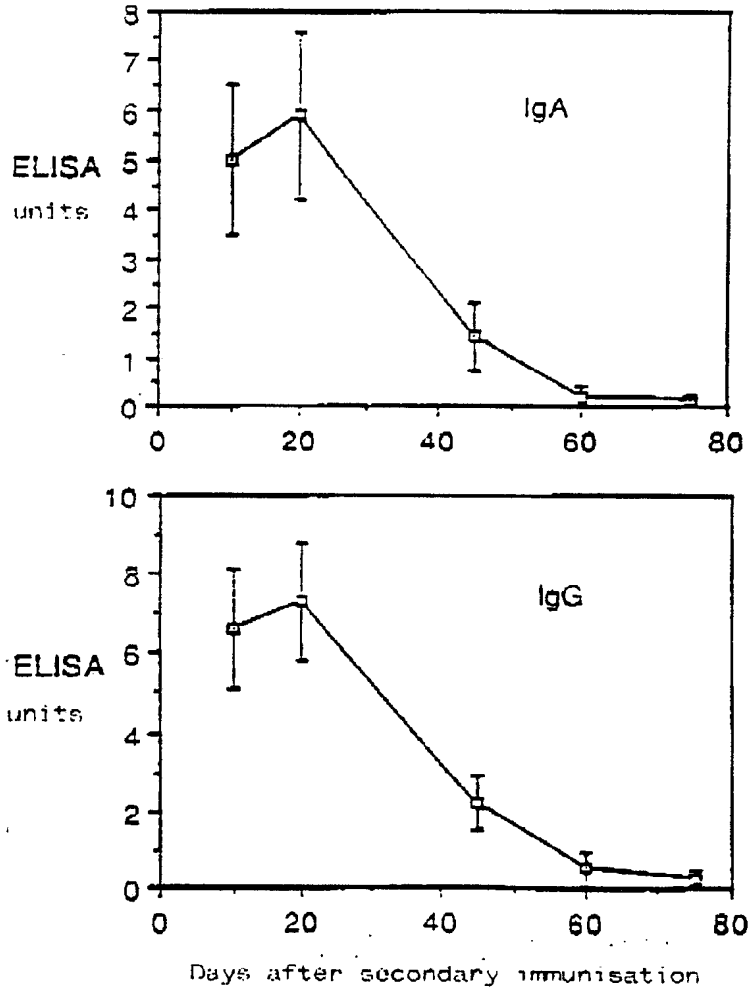
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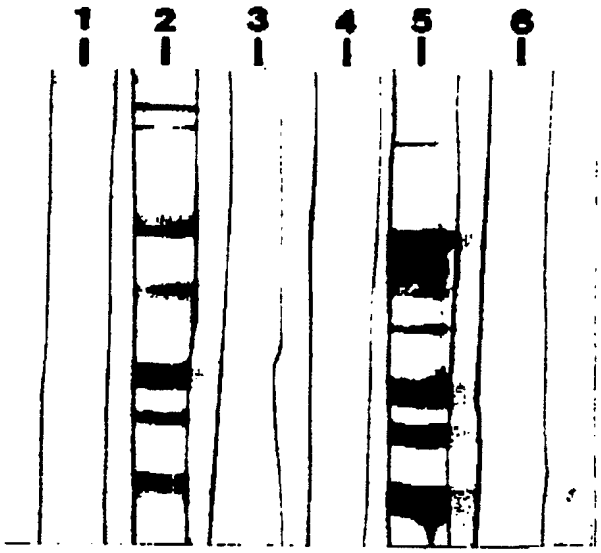
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09591

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/00, 39/12, 39/02
US CL :424/88, 89, 92
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88, 89, 92

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CA, INPADOC
search terms: liposome, pertussis, shigella, streptococcus, microcarrier, solvent dilution

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US, A, 4,199,565 (FULLERTON) 22 April 1980, see entire document.	1,2 3-9
X Y	US, A, 4,053,585 (ALLISON ET AL) 11 OCTOBER 1977, see entire document	1,2 3-9
X Y	INFECTION AND IMMUNITY, Volume 48, issued June 1985, J.V. Desiderio et al, "Immunization Against Experimental Murine Salmonellosis with Liposome-Associated-O-Antigen", pages 658-663, see entire document.	1 2-9
X Y	NATURE, Volume 252, issued 15 November 1974, A.C. Allison et al, "Liposomes as Immunological Adjuvants", page 252, see entire document.	1 2-9
X Y	INFECTION AND IMMUNITY, Volume 52, Number 2 issued May 1986, D. Wachsmann et al, "Serum and Salivary Antibody Responses in Rats Orally Immunized with Streptococcus mutans Carbohydrate Protein Conjujate Associated with Liposomes", pages 408-413, see entire document.	1-2,8 3-7,9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
07 January 1993

Date of mailing of the international search report
29 JAN 1993

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09591

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF EXPERIMENTAL MEDICINE, Volume 171, issued January 1990, R.D. Shahin et al, "Characterization of the Protective Capacity and Immunogenicity of the 69-kD Outer Membrane Protein of Bordetella pertussis", page 252, pages 63-73.	3,6,8,9
Y	WO, A, 89/11850 (FOUNTAIN) 14 DECEMBER 1989, entire document.	1-9
Y	S.A. Plotkin et al, "VACCINES" published 1988 by W.B. Saunders (PA), pages 74-97, entire document.	2,4,5
X Y	INFECTION AND IMMUNITY, Volume 54, Number 3, issued December 1986, R.L. Gregory et al, "Characterization of Immune Response to Oral Administration of Streptococcus sobrinus Ribosomal Preparations in Liposomes", pages 780-786.	1,2,8 3-7,9